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Hepatoma Ferritin in the Tissue and Serum

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SUMMARY

Ferritins from human hepatoma, normal liver and spleen, and placenta were isolated and characterized on isoelectrofocusing. Hepatoma and placenta ferritins showed similar acidic mobilities whereas spleen and liver ferritins exhibited a more basic profile. Iron to protein ratio of hepatoma ferritin was considerably low as compared with normal liver ferritin, indicating not only structural but also functional differences exist between hepatoma and normal liver ferritin. Immunological examinations, however, failed in demonstrating the antigenic specificity of hepatoma ferritin. For cliniacl oncology, it is of particular importance to find tumor associated substances, in this case ferritin, in the blood of patients with malignancies. Therefore serum ferritin concentrations in hepatoma patients were measured by radioimmunoassay. In most of patients with hepatoma, serum ferritin levels were elevated above the normal range (10~153 ng/ml) with a mean value of 427 ± 251 ng/ml. Serum ferritin concentrations were also estimated in rats with chemically induced hepatomas. Serum ferritin in rats during carcinogenesis showed a characteristic appearance which consisted of an early transient elevation and a second persistent elevation. A similar observation is well known for the occurrence of α_1-fetoprotein under the same pathological conditions. This observation seemed to indicate that tumor tissue is indeed producing the serum ferritin. However, the actual ferritin concentration in hepatoma tissues were rather lower than that in normal liver. Possible mechanisms for the elevation of serum ferritin in hepatoma were disscussed.

INTRODUCTION

Ferritin is a major iron storage protein which is found in all tissues, but particulary in high concentrations in the liver, spleen and bone marrow.

Structurally, the ferritin molecule consists of an approximately spherical protein shell of apoferritin, mol. wt. 450,000, within which variable amounts of inorganic iron is sequestered[1]. Apoferritin is presently thought to con-

sist of 20 or 24 chemically identical subunits[2], but recent evidences of multiple subunit types[3~6] indicates a considerably more complex structure.

An increasing amount of evidences suggests that ferritin exists in different structural and metabolic forms (isoferritin) in different tissues[7~10]. In addition to the isoferritins found in normal cells, of particular interest was abnormal ferritins isolated from malignant cell lines[11], rat and human hepatoma[12,13], and pancreas carcinoma[14]. This tumor isoferritin was recently designated as "Carcinofetal isoferritin" since it was also identified in fetal tissue in an early period of gestation[13].

One important aspect of carcinofetal protein is its clinical application as a tumor associated substance which can be measured in the blood of patients with malignancy.

We have recently demonstrated a high level of serum ferritin in patients with malignant diseases using a sensitive radioimmunoassay technique and proved that it has obvious potential for serological diagnosis of malignancy[15].

In the present paper, we have extended these observations in an attempt to investigate the mechanisms for elevation of serum ferritin in malignancy, particularly in hepatoma.

METHOD AND MATERIAL

Purification of tissue ferritin

The tisssue was homogenized in distilled water in a Waring Blendor and diluted with 4 ml of water per gram of tissue. The homogenate was heated in a water bath with stirring for 15 minutes at 75°C. After centrifugation at 10,000 g for 30 min, the supernatant was passed through a Millipore filter (pore size 0.45 μm).

Ferritin fraction was precipitated from this solution with $(NH_4)_2\ SO_4$ (50% saturation) and the precipitated protiein was dissolved in a smaller volume of 0.02 M pH 7.0 phosphate buffer. The solution was subsequently subjected to gelfiltration on a Sephadex G-200 column and the fractions which were eluted immediately after the void volume, were collected. The final step of purification was carried out on Sepharose 6B gelfiltration. The purity of ferritin was examined by polyacrylamide gel electrophoresis at pH 8.5.

Animals

Male Wister rats (150–200 g body weight) were housed in groups of 3 in wire mesh cages and maintained on a semisynthetic diet containing 0.06% 3′-methyl-diethylaminoazobenzene (Oriental Yeast Co, Ltd. Tokyo, Japan). Each group of rats were sacrificed every other week for 20 weeks and serum

ferritin concentrations were estimated by radioimmunoassay (RIA), for rat ferritin.

Isoelectric focusing

In order to compare the mobility of various ferritin samples under the same electrophoretic condition, a slab gel was specially devised for isoelectric focusing. Separating gel with a concentration of 3.75% acrylamide containing 2% ampholine, pH 4–6, (LKB Sweden) was sustained between thin layers of 7.5% ordinary polyacrylamide gel (Fig. 1). The top thin layer gel (applying-gel) was made not only for condensation of sample bands but also for separation of higher aggregated ferritin from monomeric ferritin[16] by the sieving effect of polyacrylamide. The bottom layer was placed to support the soft separating-gel during electrophoresis. Ferritin samples containing 20–30 μg protein in 0.02–0.03 ml, pH 7.0 phosphate buffer were applied to the wells in the applying gel and electrophoresis was performed vertically with a constant current of 12 mA for 6 hours using an apparatus purchased from Pharmacia Co, Sweden (model GE–4). The gel was stained for protein with coomassie brilliant blue G–250 and destained stepwisely according to the method of Vesterberg[17].

Chemical determination of protein and iron concentration of ferritin

Protein was determined by the method of Lowry[18] with bovine serum albmin used to obtain a calibration curve. A modification of Hill's bipyridyl method[19] for estimation of iron in the ferrous state was chosen, with Na_2

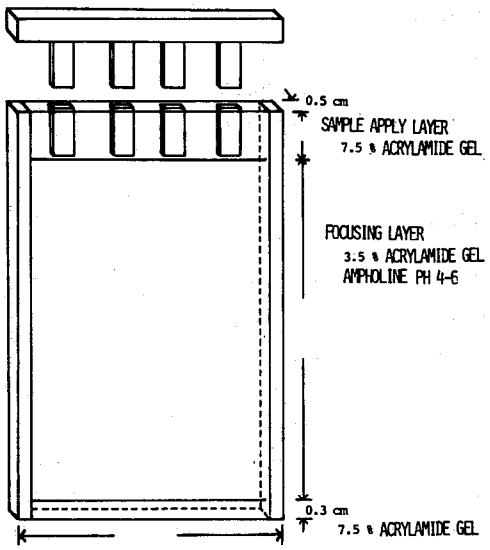

Fig. 1. Schematic drawing of slab gel electrofocusing.

SO_3 as the reducing agent.

Radioimmunoassay of ferritin

Human and rat liver ferritin were iodinated using chloramine-T and Na ^{125}I. The iodination shcedule in details was described in a previous report[15]. Although there is no exposed tyrosyl residue on the surface of the ferritin molecule[20], actual iodination occurred by chloramine-T method without any chemical modification of the molecule. For the separation of antibody bound and free ^{125}I ferritin, the double antibody method was used. The lowest ferritin concentration determined in this assay was 5 ng per ml.

Subjects and serum

Serum ferritin concentrations were estimated in 20 normal adults, 18 patients with acute hepatitis, 12 patients with chronic liver disease and 10 patients with hepatoma. All serum samples were kept at −20°C unless they used immediately for experiments.

Double immunodiffusion tests of ferritin

Immunological tests were carried out by the Ouchterloney double immunodiffusion method in 1% purified agar. The antisera against normal human liver ferritin and hepatoma ferritin were obtained from rabbits immunized by repeated subcutaneous injections of highly purified ferritin protein mixed with complete Freund's adjuvant.

RESULTS

Isoelectrofocusing of hepatoma ferritin

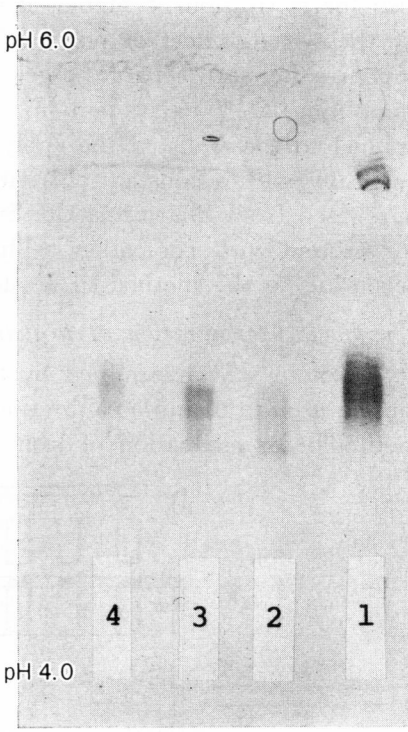

Fig. 2. Gel electrofocusing profile of ferritins from liver (1), hepatoma (2), placenta (3) and spleen (4).

Fig. 2 shows the polyacrylamide slab gel electrofocusing profile of human hepatoma ferritin compared against ferritins from normal adult liver, spleen and placenta. At least four distinct bands were revealed in all ferritin preparations. This microheterogeneity in the ferritin molecule within a single organ has been extensively studied with horse spleen ferritin[21~23]. Several bands were common to most tissues and at least two were common in all tissues. However, the relative amounts of the various bands differed

Table 1. *Iron to protein ratio of normal and tumor ferritin*

normal ferritin	0.090
tumor ferritin	0.059

Fig. 3. Double immunodiffusion test for normal liver ferritin (1) and hepatoma ferritin (2) using antisera against normal liver ferritin (A) and hepatoma ferritin (B).

in each tissue. In human hepatoma, the presence of isoferritins of a more acidic mobility than those of normal adult liver and spleen were evident. Similar acidic isoferritin profile was found in placenta tissue.

Iron to protein ratio of hepatoma ferritin

As compared with normal liver ferritin, iron to protein ratio was considerably low in hepatoma ferritin (Table 1), probably reflecting the difference in affinity of protein to iron.

Immunological analysis of hepatoma ferritin

As shown in Fig. 3, the serological specificity of human hepatoma ferritin was not distinguishable from normal liver ferritin even though antisera against normal ferritin and hepatoma ferritin were both tested against each other.

Serum ferritin concentration in patients with hepatoma

Serum ferritin concentration in various liver diseases were estimated by RIA technique (Fig. 4). In healthy adults the mean concentration of ferritin in serum was 77.5 ± 38.5 ng/ml with a range of 10 ng/ml to 153 ng/ml. All 18 patients with acute hepatitis were found to have concentrations above the normal range with a mean value of 503.7 ± 237.6 ng/ml. In most of the cases of acute hepatitis, alteration in serum transaminase level were

accompanied by proportional changes in serum ferritin (Fig. 5). The result indicates that elevation of serum ferritin in acute hepatitis is attributable to the release of ferritin from the damaged liver. In patients with chronic hepatitis and cirrhosis, serum ferritin was normal except for 2 patients with chronic active hepatitis.

In most patients with hepatoma, ferritin levels were elevated above the normal range with a mean value of 427.7±251 ng/ml. A possible mechanism for the elevation of serum ferritin in hepatoma will be discussed later.

Serum ferritin concentration in rats with chemically induced hepatoma

Serum ferritin concentrations in rats during the course of carcinogenesis was estimated by RIA (Fig. 6). A transient elevation of serum ferritin was observed at 4–8 weeks after feeding the animals with carcinogen. The second and persistent elevation occurred after 12 weeks.

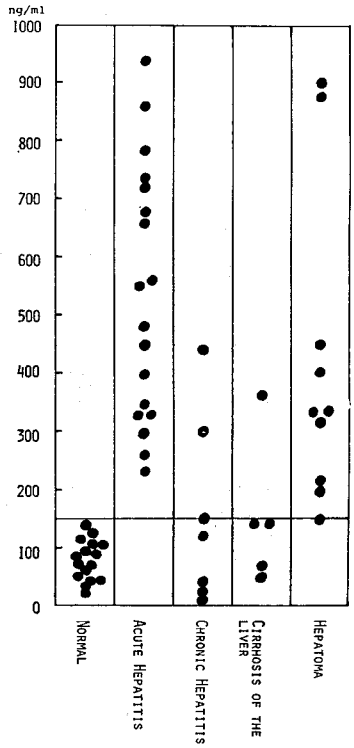

Fig. 4. Serum Ferritin concentration in various liver diseases.

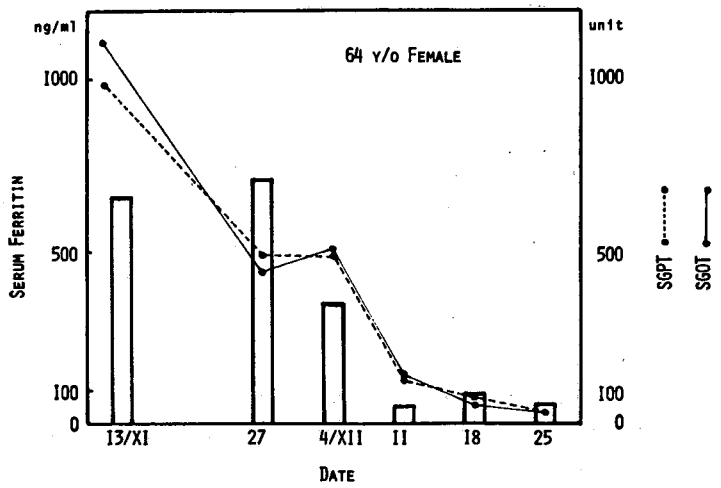

Fig. 5. Alteration of serum ferritin level during the clinical course of acute hepatitis.

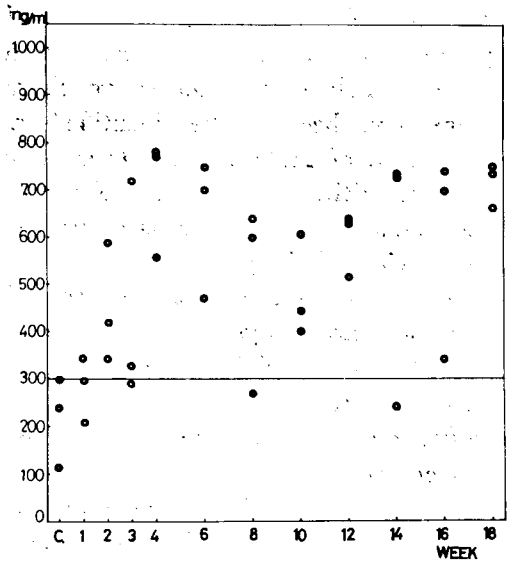

Fig. 6. Appearance of serum ferritin in rats with chemically induced hepatoma.

Table 2. *Ferritin concentration in the hepatoma tissues*

	Tissue ferritin μg/wet tissue gramm	Serum ferritin ng/ml	AFP (electrosyneresis)
Normal liver	1016.4	76.5	(−)
Hepatoma A	774.4	900.0	(+)
Hepatoma B	222.6	150.0	(+)

Ferritin concentration in human hepatoma tissues

In order to examine whether high serum ferritin levels in patients with hepatoma are associated with increased synthesis of the protein by tumor cells, ferritin concentrations in hepatoma tissues were estimated by RIA (Table 2). Unexpectedly, ferritin concentrations in hepatoma tissues were rather depleted as compared with that in normal liver, although in each hepatoma patient, serum ferritin concentration appeared to correlate with the amount of ferritin in corresponding hepatoma tissue.

DISCUSSION

In 1964, Richter first described an unique component of ferritin with rapid electrophoretic mobility in neoplastic cultured cell lines[24]. Since then, his observation has been confirmed and extended by several investigators

using either ordinary disc electrophoresis[25] or electrofocusing[12~14]. We also have previously reported distinctive isoferritins with acidic isoelectric point in rat hepatomas which were induced by chemical carcinogen[12]. These acidic isoferritins have been further identified in fetal liver[13] and placenta[26] of an early period of gestation, and thus they were designated as carcinofetoplacental isoferritin. Our present observation of acidic isoferritins in human hepatoma and placenta by isoelectrofocusing was in good agreement with these previous findings.

Structural basis for acidic nature of tumor isoferritins has been recently explored by amino acid analysis, peptide mapping[27] and subunit analysis in S. D. S. gel[27] or urea gel[28]. Results so far obtained are indicative of a distinct primary structure of tumor isoferritins, and therefore posttranslational modification of the protein is not likely to be responsible for acidic nature in electrophoresis.

Iron content of tumor isoferritin also differs from normal ferritin. The ratio of iron to protein in hepatoma ferritin studied here was considerably low as compared to normal liver ferritin. Since the essential function of ferritin is storage of iron in the tissue, the above results suggest that hepatoma ferritin is, not only structurally but also functionally, different from normal liver ferritin.

Immunologically, however, the hepatoma and normal liver ferritins were indistinguishable in our present study although some quantitative difference in antibody affinity was recently claimed[27].

Despite these distinct properties, an implication of the carcinofetoplacental isoferritin is presently complicated since ferritins from some normal organs such as heart and kidney[27], were also shown to have more acidic isoelectric point than that of liver or spleen ferritins. In order to explain this phenomenon, Drysdale *et al* have recently postulated[30] that the presence of carcinofetoplacental isoferritin probably reflects a phenotypic shift in the distribution of tissue specific isoferritin form liver type to heart type in much the same manner as the case of lactic dehydrogenese on cancer[31]. Further structural analysis is, however, still required to elucidate the relationsphip between tumor isoferritins and tissue specific isoferritins.

For clinical oncology, it is an important task to find tumor associated substances, in this case serum isoferritin in the blood of patients with malignancies.

Ferritinemia in malignancy was first emphasized by Reissmann *et al*[32] using a quantitative immunoprecipitin technique. More recently Buffe *et al*[33] found an carcinofetal protein (α_2H protein) which was lately identified as belonging to the ferritin class of proteins, in the sera of 80% of children

and 50% of adults with malignant diseases.

Early studies of serum ferritin, however, have used relatively crude assay methods. The recent introduction of radioimmunoassay techniques has allowed us more accurate and sensitive measurement of circulating ferritin in detecting and monitoring the malignancies. We have previously demonstrated a high level of serum ferritin in patients with malignant diseases in general[15].

In the present investigation, serum ferritin concentration in patients with hepatoma were estimated by RIA and significant elevations were found in most of the patients.

Elevated concentrations of serum ferritin were also detected in rats with chemically induced hepatoma.

Furthermore, serum ferritin in rats during carcinogenesis showed a highly characteristic appearance which consists of an early transient elevation and a late persistent elevation. An analogous observation is well documented for the occurrence of α_1-fetoprotein in the serum of rat hepatomas induced by the same azo-dye carcinogen[34]. This analogy in appearence of serum ferritin and serum α_1-fetoprotein naturally led us to speculate on the possibility that hepatoma tissue is actually overproducing ferritin (possibly the carcinofetoplacental isoferritin).

In fact, an increased production of the ferritin by tumor cells has been reported in acute leukemia[35]. However, in the present study, ferritin concentrations in hepatoma tissues were unexpectedly low as compared with that of the normal liver, indicating that the mechanisms for the elevation of serum ferritin in hepatoma is probably different from that of leukemia.

Nevertheless, the possibility that the hepatoma tissue is synthesising a certain type of ferritin (possibly carcinofetal isoferritin) which is destined to be secreted into circulation and thus ferritin is not accumulated in hepatoma tissue, has not been excluded. Generally, secreted proteins are synthesised primarily on membrane bound polysomes whereas the proteins produced for intracellular purposes are synthesised primarily on free polysomes. In this context, an interesting observation is that even the normal rat liver is indeed synthesising some portion of ferritin on polysomes attached to endoplasmic reticulum[36,37].

Recently, we have examined microheterogeneity of serum ferritin on column isoelectrofocusing (unpublished). In addition to the main peak of normal ferritin, an acidic isoferritin was identified in the sera of the myelomnocytic leukemia. This result seems to suggest that at least some portion of serum ferritin in malignancy is derived from the tumor tissue itself.

As for the origin of the remaining portion of serum ferritin with a normal isoelectric point, a plausible speculation may be that it results from an increase of reticuloendothelial iron which is commonly associated with anemia of neoplastic diseases.

Supporting the above hypothesis, there are now well established evidences that in normal subjects and in patients with iron defficiency or iron overload, serum ferritin concentration are closely related to the amount of storage iron in reticuloendothelial system[38].

According to recent studies on metabolism of serum ferritin, ferritin in circulation is mostly taken up by the liver[39,40].

Therefore it may be tempting to investigate whether the increase in reticuloendothelial iron and the resulting elevation of serum ferritin in mailgnancy arises from the impraired uptake of circulating ferritin by the liver.

Furthermore, as an alternative for the origin of normal ferritin in hepatoma serum, a release from damaged tissue by metastasis or direct invasion of tumor should be also taken into account.

Consequently, the elevation of serum ferritin in malignancy seems to involve a number of different mechanisms and therefor at present its concrete interpretation should be treated with some reserve.

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